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# Forms of phosphorus in nutrition of Halobacterium Halobium and Halobacterium Salinarium

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# FORMS OF PHOSPHORUS IN NUTRITION OF *HALOBACTERIUM HALOBIUM* AND *HALOBACTERIUM SALINARIUM*

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ABSTRACT

Growth of Halobacterium halobium and Halobacterium salinarium in response to various sources of phosphorus (ortho-phosphate, pyrophosphate, tripolyphosphate and DL-glycerophosphate) was determined turbidometrically. The bacteria were grown in side-arm flasks that allowed direct reading of turbidity. The growth medium was synthetic medium (SM) of Grey and Pitt (1975) in which the sources of phosphorus were modified. The intent of the study was to ascertain whether a given form of phosphorous is more effective in stimulating growth of these bacteria than others, and to determine optimum levels of that form of phosphorus in the nutrient medium.

The primary conclusion drawn from this study is that only ortho-phosphate has significant nutritional value in promoting growth of H. halobium and H. salinarium. Pyrophosphate, tripolyphosphate and DL-glycerophosphate apparently provide only little or no nutritional stimulus for growth of both species.

While casamino acids have been included traditionally in complex medium used for growing halobacteria, a secondary finding in this study is that H. salinarium exhibits improved growth rates when tryptone and calcium ion are substituted for casamino acids.

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My deepest thanks go to Mr. and Mrs. James for their love, and moral support for my education.

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This thesis is dedicated to my father and mother, Mr. and Mrs. M. V. Parekh, and my family.

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INTRODUCTION:

Halobacteria, a group of photobacteria, lack chlorophyll and the usual photosynthetic apparatus. Instead, the light energy these organisms use is absorbed by bacteriorhodopsin, a chemical substance resembling visual purple (the light sensitive pigment in the human retina). The absorbed light energy is used to generate ATP needed for their growth (Nester, et al. 1978). Halobacteria can live only in very strong salt solutions, such as salt ponds and brines used for curing fish on which they may produce red patches of growth. They are able to grow utilizing chemical energy from organic material, but only in the presence of oxygen; under anaerobic conditions they rely on their light-trapping pigment for energy (Stoeckenius 1964, Danon and Stoeckenius 1974, Nester, et al. 1978). They are aerobic and grow at a temperature between 37° C - 45° C (Gibbons and Payne 1961, Gibbons 1969).

There are two types of halophilic bacteria, the moderate halophiles and the extreme halophiles. The moderate halophiles requires 3 - 15% salt concentrations for growth while the extreme halophiles require 15% or higher (Gibbons 1969). Morphologically, halophilic bacteria are divided into two groups, Halobacterium, which is rod shaped, and Halococcus, which is spherical (Breed, et al. 1957). Most nutritional and physiological studies have been done with Halobacterium.

Comparatively, Halobacterium is more sensitive than Halococcus to changes in salt concentrations; thus, cellular lysis of Halobacterium occurs at lower salt concentrations (Kushner 1968).

Interest in studying these organisms developed because halophilic bacteria spoil fish, bacon and hides (Lochhead 1934, Browne 1947). Subsequent interest in studying these organisms was directed toward their basic physiology because of their high salt requirement (3-25% salt) compared to the physiological salt requirement (0.9% salt) for most organisms (Gibbons and Payne 1961, Gibbons 1969). Since these organisms require high levels of salt in the medium, they are considered adapted to a severe environment, and presumably depend on many specialized biological events for their growth.

Halophilic bacteria are difficult to culture because of their high salt requirement (Buchanan 1918). Development of a chemically-defined medium for growth of halophilic bacteria is important for further studies on the metabolism of these organisms. Although some attempts have been made to develop a chemically-defined medium for the halobacteria (Weber 1949, Dussault and Lachance 1952, Katznelson and Lochhead 1952, Browne and Gibbons 1955, Sehgal and Gibbons 1960, Dundas, et al. 1963, Onishi, et al. 1965, Grey and Pitt 1975), such a growth medium, which also promotes rapid growth of these organisms, has not been designed. Thus, studies of the various physiological and enzymatic components, specialized or not, in halobacteria have

been difficult and often inconclusive (Bayley and Gibbons 1956, Weber 1949). Earlier workers (Buchanan 1918, Larrison and Kennedy 1922, Lochhead 1934, Gibbons 1936) cultured halobacteria on complex media containing fish infusion, yeast extract, starch or milk; however, detailed investigation of the nutritional requirements of halobacteria has awaited the development of an adequate chemically-defined medium. Weber (1949) described a synthetic medium which allowed some growth but required the addition of gelatine hydrolyzate for heavy growth. Although a high level of magnesium was said to favor growth, no data on the essential nature of this or any other constituent of the medium were included. Katznelson and Lochhead (1952) found that vitamins, purines and pyrimidines were not essential for growth of red halophiles, but that both yeast extract and its ash produce considerable stimulation. Brown and Gibbons (1955) studied the effect of magnesium, potassium and iron on the growth and morphology of red halophilic bacteria. They found that potassium ion was essential for growth of the red halophiles (Halobacterium cutirubrum, Halobacterium halobium, Halobacterium salinarium and Sarcina littoralis) in a medium containing 1.5% casamino acids and 0.25% glutamic acid. From 0.05 to 0.1 mg/ml potassium ion was satisfactory for maximum growth. Magnesium, sodium and chloride ions are required to maintain cell structure and rigidity. As their concentration is reduced, the cell wall dissolves and cell membrane breaks up into tiny fragments. The intracellular ion is mainly potassium

which is required to maintain structural integrity of the ribosomes and for protein synthesis. This ion is also important in maintaining activity of intracellular enzymes.

Halobacterium halobium and Halobacterium salinarium do not metabolize carbohydrates (Buchanan and Gibbons 1974). Instead, amino acids from the medium are used as the energy and carbon source in aerobic conditions (Katznelson and Lochhead 1952), presumably utilized via Kerb's cycle and terminal respiration to yield ATP through oxidative phosphorylation. In anaerobic conditions, photophosphorylation via the photosynthetic membrane is the rule (Stoeckenius 1965, Danon and Stoeckenius 1974).

Potential effects of phosphorous levels and types of phosphorous compounds on growth of these organisms in either aerobic or anaerobic conditions apparently have not been considered. In attempts to establish a defined culture medium, a modification of the synthetic medium of Onishi, et al. (1965), synthetic medium of Grey and Pitt (1975) has been used often as a base for developing adequate growth of halobacteria. The synthetic medium of Grey and Pitt (1975) contains three times higher phosphate concentration than that of Onishi, et al. (1965). In this medium only L-amino acis are used and ammonium chloride and nucleotides are omitted from it. The potassium ion concentration is the same (1 mg/ml) as recommended by Gouchnauer and Kushner (1972) for optimum growth and pH of 6.6 rather than 6.2 recommended by Onishi, et al. (1965). Grey and

Pitt (1975) showed that the nucleotide fraction is not needed in the medium since it only serves as an additional source of phosphate in medium deficient in phosphate. The objective of this study then is to determine the differential effects of various forms of phosphorus (ortho-phosphate, pyrophosphate, tripolyphosphate, and glycerophosphate) on the growth of Halobacterium halobium and Halobacterium salinarium, in an attempt to enhance growth rates of these organisms in various culture media in aerobic condition.

## MATERIALS AND METHODS:

### A. Source and Isolation of Bacterial Organisms:

*Halobacterium salinarium* (Harrison and Kennedy 1922).

The organism was obtained from the laboratory of Dr. Dalton Harrington, Professor of Biology, California State College, San Bernardino, and had originally been isolated from the surface of a highly saline pond at the California State Universities and Colleges Desert Studies Center at Soda Springs, Mojave Desert, California (Odebela 1979). This organism grows with a thick surface crust of orange-red salts with an orange-red solution under it during the summer season. The bacteria are found primarily inside the salt crystals and not in the water. This pond looks orange-red with no crystals during winter and spring seasons. The bacteria in this case, are found primarily in the mud at the bottom of the pond rather than suspended in the solution.

A partial chemical analysis of the red solution was conducted using standard procedures as provided by the Hach Chemical Company.

Isolation procedures followed those of Eimhjellen (1965). Flasks containing fish-peptone broth and 2.75 grams of pink colored salt crystals were incubated at 37° C in a shaking water bath until a red turbidity was evident (9 days). The cultures

were centrifuged for 20 minutes at 5000xg, the cells resuspended in sterile 25% sodium chloride solution (5 ml). For initial plating, fish-peptone-agar plates were streaked for isolation with 0.2 ml aliquots of centrifuged culture. The plates were incubated at 37°C in sealed plastic bags. After 12 days of incubation, a number of colonies showing differences in pigmentation (pink to reddish) were observed. These colonies were subcultured on fresh fish-peptone-agar plates to isolate pure colonies and incubated as above. After 12 days, uniform pinkish color colonies were formed. A stock culture was maintained on fish-peptone-agar slant.

Halobacterium halobium (Pelter 1931).

A freeze-dried pink color culture (salt crystals) was obtained from American Type Culture Collection. The culture was suspended in casamino acid broth (Gouchnauer, et al. 1972) and a final few drops of broth were streaked on casamino acids agar plates and slants. Pink colored colonies were apparent after seven days and were subcultured three times before using experimental media.

B. Temperature Study:

To determine at which temperature H. halobium and H. salinarium grow best, the cultures (casamino acids agar slants, tryptone agar slants and fish-peptone-agar slants) were kept at various temperatures (30° C, 32° C, 35° C, 37° C, 39° C, 40° C,

42° C, and 45° C).

C. Staining Procedure:

For the study of cellular form, the Dussault method of staining halophilic bacteria was used (Dussault 1955). One loopful of the organism was spread on a microscope slide and allowed to air dry. After being air-dried, the cells are fixed and desalted simultaneously by immersing the slide in 2% acetic acid for five minutes. The slide is removed and dried unwashed. After drying the smear is covered with 0.25% aqueous solution of crystal violet. Excess of crystal violet is removed by rinsing the slide with water. The slide is then ready for microscopic study.

D. Culture Media:

a. Fish Broth-Peptone (Lochhead 1934).

500 gm of a minced fresh cod fish was steamed in 1000 ml of tap water for one hour to prepare a fish broth. The steamed preparation was filtered through several layers of cheese cloth. The pH of the filtrate was adjusted to 8.2 with 1N KOH. The broth was diluted 1:1 with distilled water before use. To the filtrate (1000 ml) was added:

| | |
|---|---|
| Peptone (Difco) | 1.00 gm |
| Glycerol | 5.00 gm |
| NaCl | 250.00 gm |

The medium was autoclaved at 122° C and 18 lb pressure for 15 minutes. Agar (2%) was added aseptically to sterile fish broth-peptone solution to provide initial plating medium, FPA.

b. Modified Complex Medium of Sehgal and Gibbons (Gouchnauer, et al. 1972).

CM was prepared in 100 ml lots for initial plating of the organisms, and contained the following:

| | |
|---|---|
| Vitamine free Casamino Acids (Difco) | 0.75 gm |
| Yeast extract (Difco) | 1.00 gm |
| $MgSO_4.7H_2O$ | 2.00 gm |
| Sodium citrate | 0.3 gm |
| KCl | 0.2 gm |
| NaCl | 25.00 gm |

Ferrous ion ($Fe^{+2}$) was supplied by adding 1.00 ml of stock solution of 4.98% $FeSO_4.7H_2O$. The pH was adjusted to 6.8 with 1 N NaOH. The medium was sterilized at 122° C and 18 lb pressure for 15 minutes. Aseptically, (2%) agar was added to sterile solution to provide a plating medium, CMA.

Further modification of CM in this study consisted of substituting tryptone (Difco) for casamino acids and adding $CaCl.6H_2O$ and removing sodium citrate. The addition of calcium chloride was prompted by the result of the qualitative chemical analysis of the solution from which H. salinarium had been isolated. The exact composition of this modified culture medium, TM, is listed below

c. Composition of TM:

| | |
|---|---|
| Tryptone | 0.25 gm |
| Yeast extract (Difco) | 1.00 gm |
| NaCl | 25.00 gm |
| $MgSO_4.7H_2O$ | 2.00 gm |
| KCl | 0.20 gm |
| $CaCl_2.6H_2O$ | 0.20 gm |

The final volume was made to 100 ml with distilled water. The pH was adjusted to 7.1 with 1N NaOH. Agar (2%) was added aseptically to provide a plating medium, TMA.

In addition to testing the growth response of H. halobium and H. salinarium to calcium chloride, both species were inoculated in TM without calcium chloride and CM with calcium chloride.

d. Synthetic Medium of Grey and Pitt (1975), containing ortho-phosphate:

The composition of 100 ml lots of SM is listed below:

| | | |
|---|---|---|
| L-alanine | 43 | mg |
| L-arginine | 40 | mg |
| L-cystenine.HCl.$H_xO$ | 5 | mg |
| L-glutamic acid | 130 | mg |
| Glycine | 6 | mg |
| L-isoleucine | 44 | mg |
| L-leucine | 80 | mg |

| | |
|---|---|
| L-methionine | 37 mg |
| L-serine | 61 mg |
| L-phenylalanine | 26 mg |
| L-proline | 5 mg |
| L-threonine | 50 mg |
| L-tyrosine | 20 mg |
| L-valine | 100 mg |
| $CaCl_2.7H_2O$ | .07 mg |
| $CuSO_4.5H_2O$ | 5 µg |
| $FeCl_2.4H_2O$ | .23 mg |
| KCl | 100 mg |
| $KH_2PO_4$ | 15 mg |
| $K_2HPO_4$ | 15 mg |
| $KNO_3$ | 10 mg |
| $MgSO_4.7H_2O$ | 2.00 gm |
| $MnSO_4.H_2O$ | 30 µg |
| NaCl | 25.00 gm |
| Sodium citrate | 50 mg |
| $ZnSO_4.7H_2O$ | 44 µg |
| Glycerol | 0.1% w/v |

The ingredients were obtained from separate stock solutions (1% w/v) of each amino acid and of the minor elements and combined as required. The pH was adjusted to 6.6 with 1N NaOH. The medium was autoclaved at 122° C and 18 lb pressure for 15 minutes. It is referred to as SM in this study. This medium

was further modified into basal synthetic medium potassium by adding Kcl in the amount of 200 mg/100 ml of SM.

e. Modified Synthetic Medium, SM, I:

This medium contained pyrophosphate in place of $K_2HPO_4$ and $KH_2PO_4$. Four concentrations were prepared by incorporating pyrophosphate ($Na_4P_2O_7 \cdot 4H_2O$) in the following amounts, 10 mg, 15 mg, 20 mg, and 30 mg/100 ml. The pH was adjusted to 6.6 with 1N NaOH and autoclaved at 122° C and 18 lb pressure for 15 minutes. It is referred to as SM I in this study.

f. Modified Synthetic Medium, SM, II:

This medium contains tripolyphosphate in place of $K_2HPO_4$ and $KH_2PO$. Four concentrations were prepared by incorporating tripolyphosphate ($Na_5P_3O_{10} \cdot 10H_2O$) in the following amounts: 10 mg, 15 mg, 20 mg, and 30 mg/100 ml. The pH was adjusted to 6.6 with 1N NaOH and autoclaved at 122° C and 18 lb pressure for 15 minutes. It is referred to as SM II in this study.

g. Modified Synthetic Medium, SM, III:

This medium contains glycerophosphate in place of $K_2HPO$ and $KH_2PO$. Four concentrations were prepared by incorporating glycerophosphate in the following amounts: 10 mg, 15 mg, 20 mg, and 30 mg/100 ml. The pH was adjusted to 6.6 with 1N NaOH and autoclaved at 122° C and 18 lb pressure for

15 minutes.

E. Method of Determination of Growth in Various Media:

Growth rates of H. halobium and H. salinarium were studied by incubating 0.2 ml aliquots of centrifuged culture in side-arm flasks containing various media at 37° C in shaker water bath for five days. Optical densities (turbidities) were measured by Klett meter at 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs during the five days growth period.

RESULTS:

Based on culture conditions and microscopic observation of stained cells and fresh cells suspended in all media, H. halobium and H. salinarium isolated in this research are gram negative, aerobic, non-motile and rod-shaped.

The requirement of 25% to 30% NaCl for growth confirms that both these species are extremely halophilic. The standard NaCl concentration used in this study was 25%.

Halobacterium halobium and H. salinarium grow well at 37° C in CMA or TMA plates, in consistence with reported optimum temperature for other strains of the genus (Gauchnauer and Kushner 1969). When plates of TMA were streaked with both species, the best growth was at temperatures of 32° C to 37° C. When plates of FPA were streaked with both species, the best growth was at 32° C to 35° C. On FPA plates no growth was observed above 35° C (Table 1).

TABLE 1: Visual estimates of growth of both species at various temperatures during five days of culture

| Media | Temperature | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 30°C | 32°C | 33°C | 34°C | 35°C | 37°C | 39°C | 40°C | 42°C | 45°C |
| FPA[a] | -- | ---- | ----- | ---- | ---- | - | - | - | - | - |
| CMA[b] | - | - | -- | -- | -- | ----- | -- | -- | - | - |
| TMA[c] | -- | --- | --- | --- | ---- | ----- | -- | -- | - | - |

- = No growth
-- = Growth is very slow
--- = Moderate growth
---- = Good growth
----- = Optimum growth

a = Fish Peptone Agar
b = Complex Medium Agar
c = Tryptone Medium Agar

Qualitative analysis of the solution from which H. salinarium was isolated showed the presence of several inorganic ions (Table 2). The presence of a pronounced level of calcium ion provides a contrast to the relatively low amounts of this ion incorporated in the various defined media used to study growth of the halobacteria.

Table 2: Qualitative analysis of the solution from which H. salinarium was isolated:

| Inorganic ions | Presence or absence |
|---|---|
| NaCl | + |
| $MgSO_4$ | + |
| $CaCl_2$ | + |
| KCl | + |
| $MnSO_4$ | + |
| $Fe^{+2}$ | - |

\+ = Presence of ions in the solution

\- = Absence of ions in the solution

Growth of H. halobium and H. salinarium in the CM with and without calcium chloride and in the TM with and without calcium chloride:

Both CM and TM with or without calcium chloride allowed the same rapid growth for H. halobium (Tables 3 and 4; Figures 1 and 2). However, on the other hand, TM with calcium allowed more rapid growth than in TM without calcium, and in CM with or without calcium for H. salinarium (Tables 5 and 6; Figures 3 and 4).

TABLE 3: Growth of the H. halobium in CM with and without calcium.

(Mean reading of two replications)

| Time in hours | Optical density with calcium | Optical density without calcium |
|---|---|---|
| 24 | 0.294 | 0.300 |
| 48 | 1.185 | 1.200 |
| 72 | 1.790 | 1.800 |
| 96 | 1.960 | 2.000 |
| 120 | 1.965 | 1.980 |

TABLE 4: Growth of *H. halobium* in TM with and without calcium.

(Mean reading of two replications)

| Time in hours | Optical density with calcium | Optical density without calcium |
|---|---|---|
| 24 | 0.290 | 0.300 |
| 48 | 0.180 | 1.200 |
| 72 | 1.800 | 1.825 |
| 96 | 2.000 | 1.900 |
| 120 | 2.000 | 2.000 |

TABLE 5: Growth of *H. salinarium* in CM with and without calcium.

(Mean reading of two replications)

| Time in hours | Optical density with calcium | Optical density without calcium |
|---|---|---|
| 24 | 0.185 | 0.245 |
| 48 | 0.870 | 0.970 |
| 72 | 0.925 | 1.050 |
| 96 | 1.420 | 1.580 |
| 120 | 1.465 | 1.605 |

TABLE 6: Growth of H. salinarium in TM with and without calcium.

(Mean reading of two replications)

| Time in hours | Optical density with calcium | Optical density without calcium |
|---|---|---|
| 24 | 0.300 | 0.199 |
| 48 | 1.200 | 0.910 |
| 72 | 1.820 | 0.980 |
| 96 | 2.000 | 1.200 |
| 120 | 2.000 | 1.450 |

Based upon the close similarity in slope of the growth curves in both media (CM and TM) with and without calcium between 24 - 72 hours after inoculation, rates of growth of H. halobium in CM and TM, with and without calcium are approximately the same (Figures 1 and 2). There is no shortening of the lag phase of growth in either media with or without calcium.

With optical density plotted against time, the rate of growth of H. salinarium in TM with calcium is better than in TM without calcium, in CM with and without calcium (Figures 3 and 4). This conclusion is based upon the observed slope of growth curve, which is greater in TM with calcium than in the other media, between 24 and 72 hours incubation time after inoculation. However, there is no shortening of the lag phase of growth in both media with or without calcium.

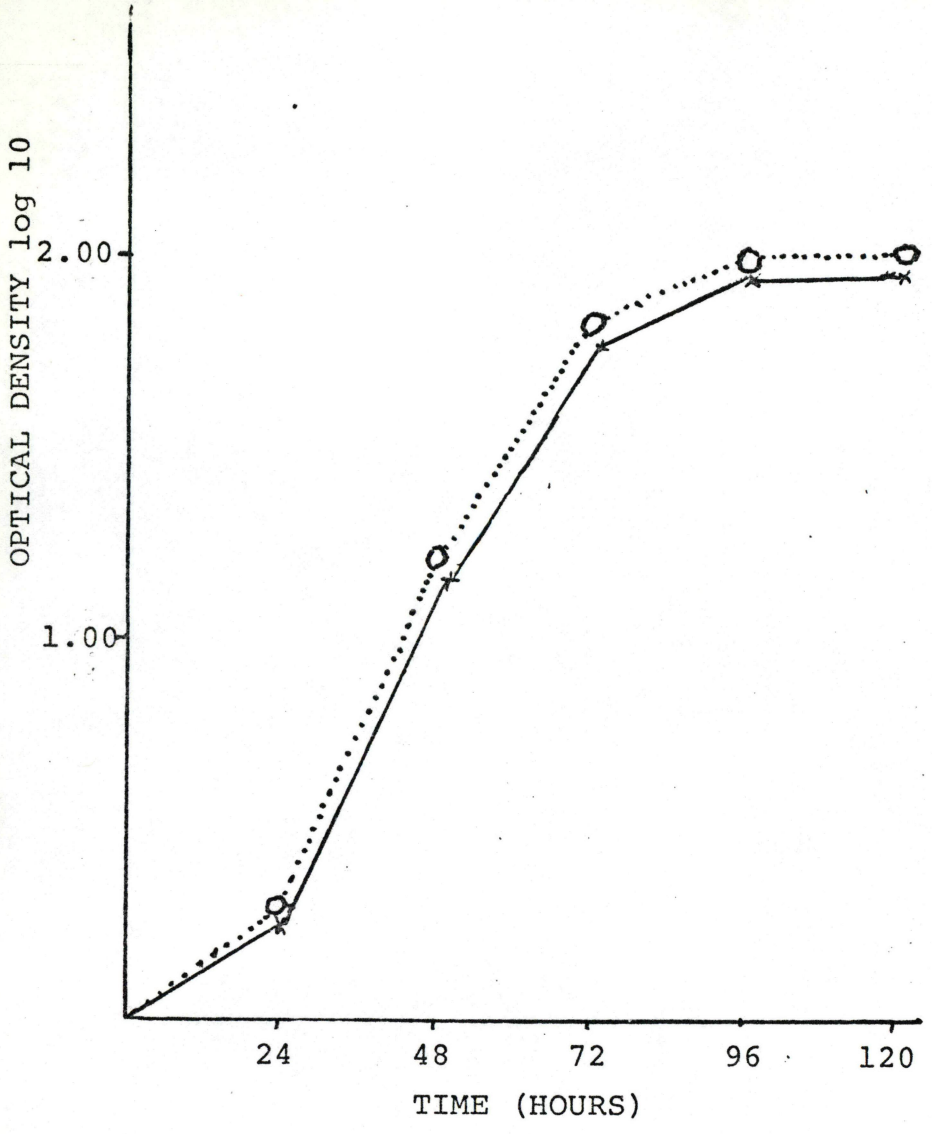


Figure 1. Growth curves of Halobacterium halobium in CM with, O····O, and without, X——X, calcium.

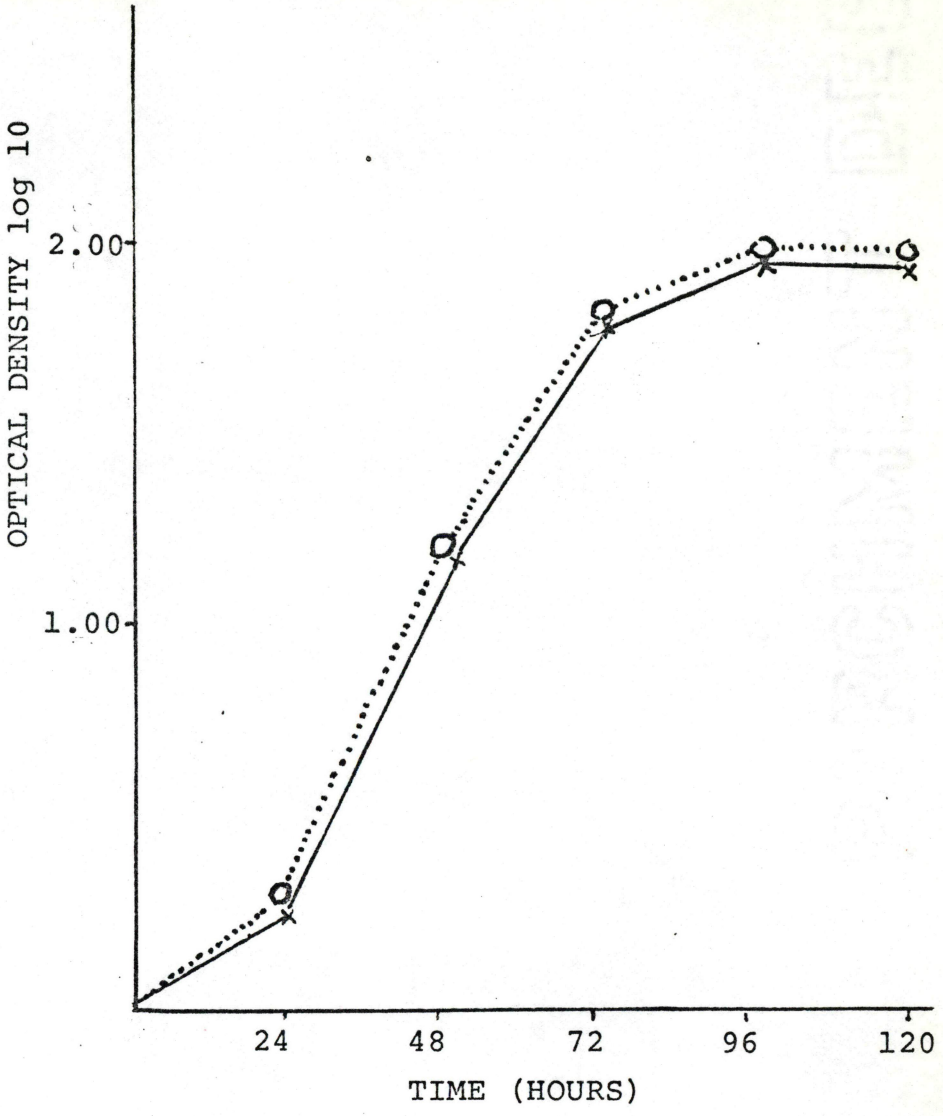


Figure 2. Growth curves of Halobacterium halobium in TM with, O····O, and without, X——X, calcium.

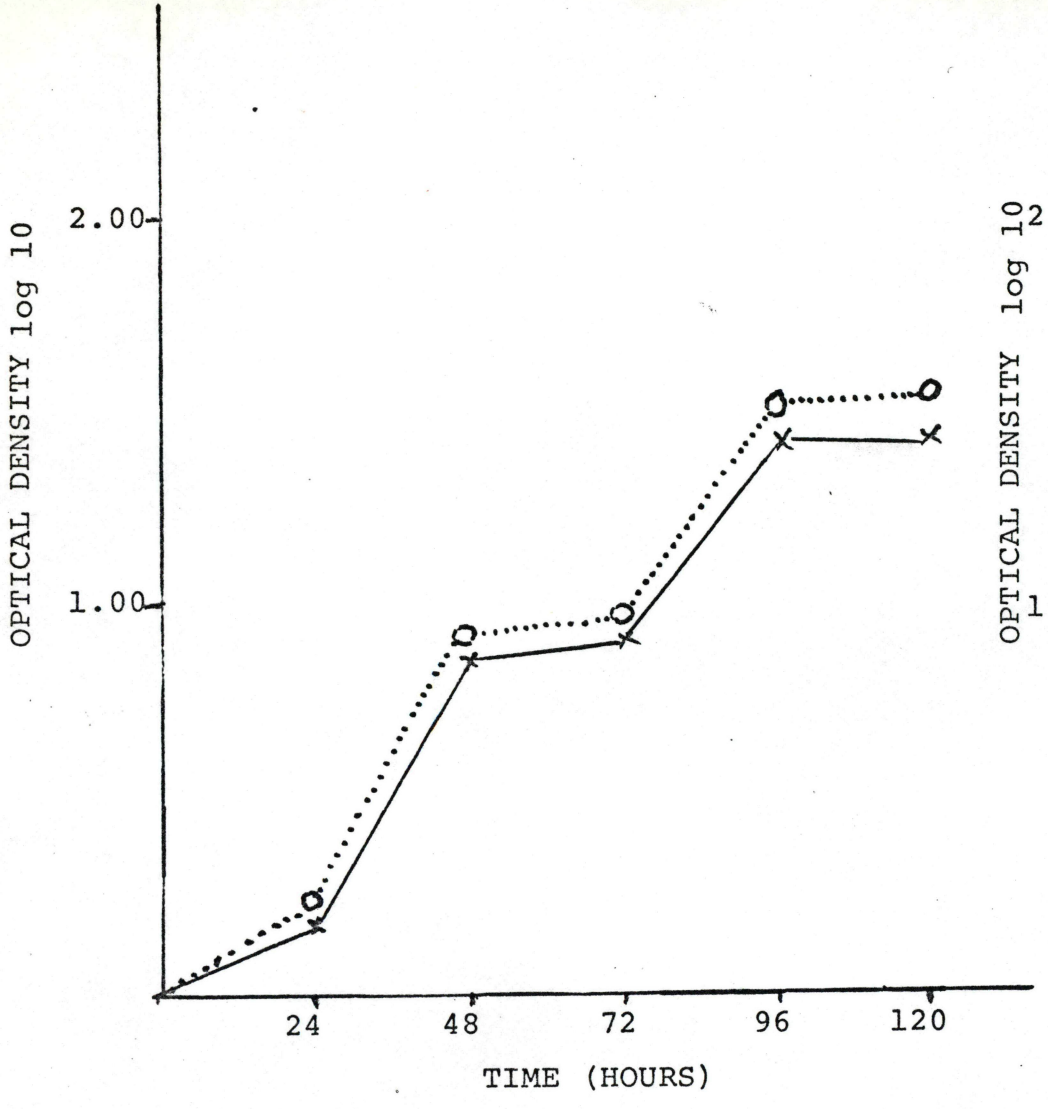


Figure 3. Growth curves of Halobacterium salinarium in CM with, O····O, and without, X——X, calcium.

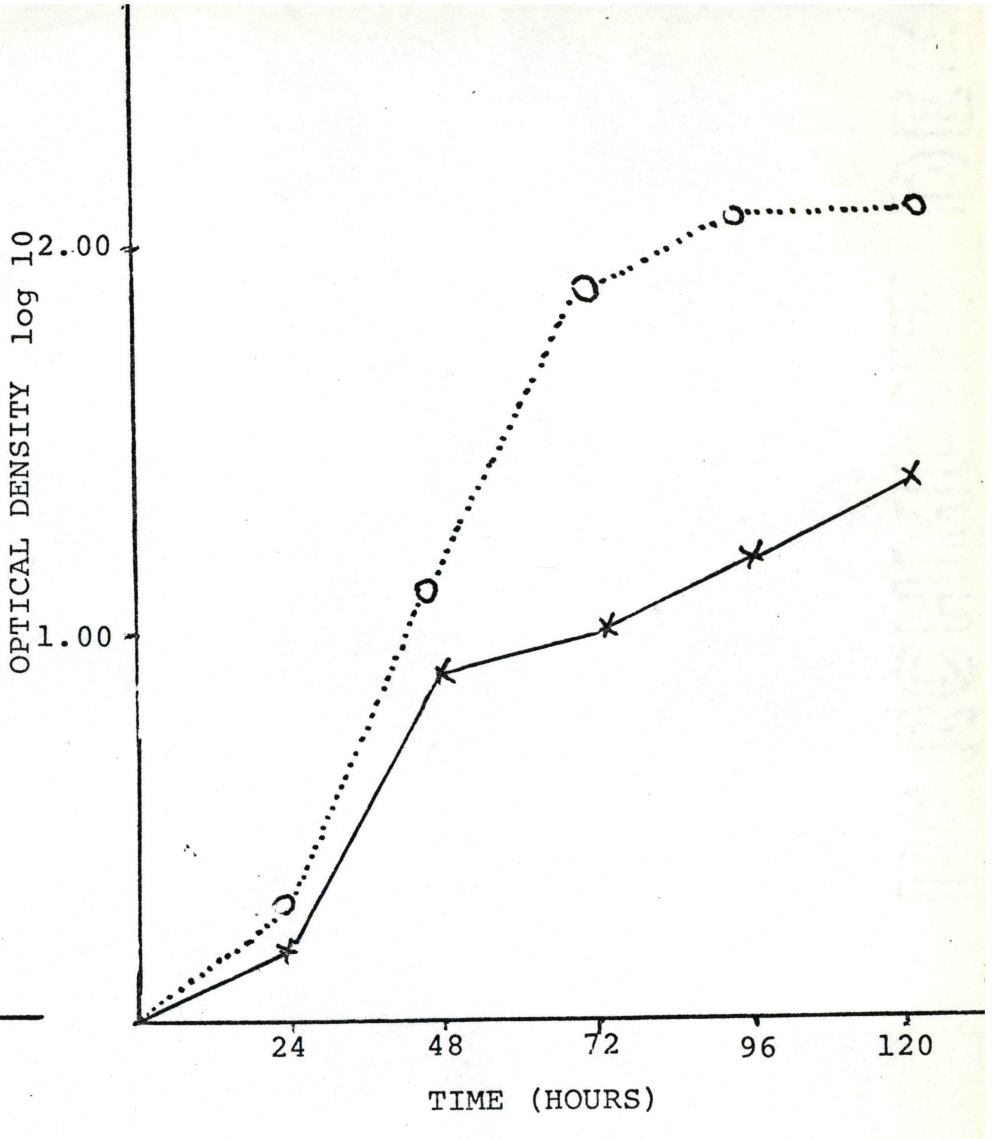


Figure 4. Growth curves of Halobacterium salinarium in TM with, O····O, and without, X——X, calcium.

Growth of H. halobium and H. salinarium in synthetic-defined media containing various forms of phosphorus:

(1) Growth of H. halobium and H. salinarium in SM, SM I, SM II, and SM III.

SM allowed rapid growth (Figure 5), giving the greater optical density for H. halobium than H. salinarium (Table 7). On the other hand, SM I allowed little growth (Figure 6) while SM II and SM III did not support any growth (Figures 7 and 8).

(2) Effect of ortho-phosphate ($K_2HPO_4$-15 mg; $KH_2PO$-15 mg/100 ml of SM).

Onishi, et al. (1965) used a relatively lower amount of ortho-phosphate (5 mg/100/ml) in their media. The addition of three times higher ortho-phosphate in SM caused marked stimulation of growth (Table 7).

(3) Effect of pyrophosphate at various concentrations ($Na_4P_2O_7.4H_2O$: 10, 15, 20, and 30 mg/100 ml).

Addition of pyrophosphate caused little stimulation of growth. Out of the four solutions of pyrophosphate used, only the 15 mg/100 ml concentration exhibited an enhancement of growth of H. halobium (Table 8 and Figure 6).

TABLE 7: Growth of Halobacterium halobium and H. salinarium in synthetic medium containing ortho-phosphate (15 mg $K_2HPO_4$; 15 mg $KH_2PO_4$/100 ml of SM.

(Mean reading of two replications)

| Time in hours | Optical Density | |
|---|---|---|
| | H. halobium | H. salinarium |
| 24 | 0.700 | 0.670 |
| 48 | 0.850 | 0.770 |
| 72 | 1.200 | 0.850 |
| 96 | 1.400 | 0.850 |
| 120 | 1.410 | 0.850 |

TABLE 8: Growth of Halobacterium halobium and H. salinarium in synthetic medium containing pyrophosphate at various concentrations of SM I.

(Mean reading of three replications)

| Concentrations pyrophosphate mg/100 ml | Time in hours | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 24 | 48 | 72 | 96 | 120 | 24 | 48 | 72 | 96 | 120 |
| | Optical Density | | | | | | | | | |
| | H. halobium | | | | | H. salinarium | | | | |
| 10 | 0.10 | 0.15 | 0.15 | 0.20 | 0.20 | 0.10 | 0.10 | 0.12 | 0.12 | 0.12 |
| 15 | 0.20 | 0.28 | 0.40 | 0.42 | 0.38 | 0.10 | 0.15 | 0.18 | 0.19 | 0.20 |
| 20 | 0.10 | 0.15 | 0.15 | 0.20 | 0.20 | 0.10 | 0.10 | 0.12 | 0.12 | 0.12 |
| 30 | 0.10 | 0.15 | 0.15 | 0.20 | 0.20 | 0.10 | 0.10 | 0.12 | 0.12 | 0.12 |

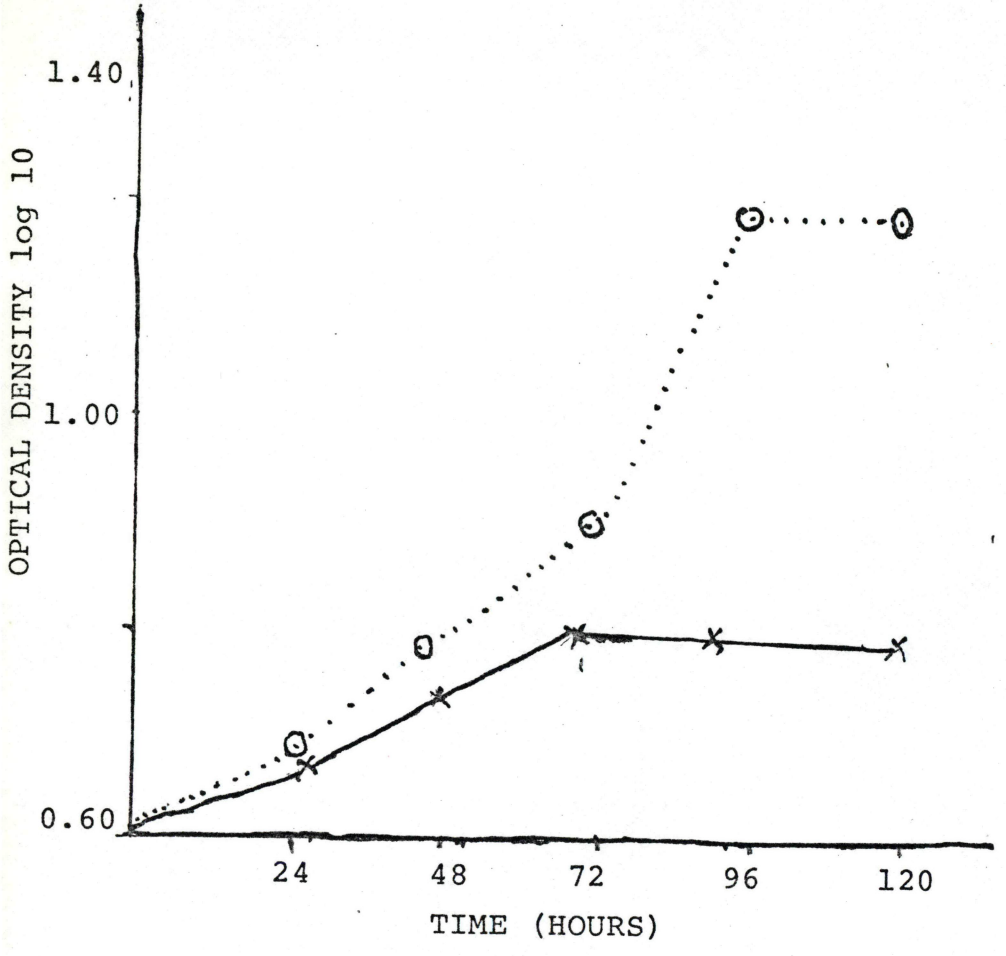


Figure 5. Growth curves of Halobacterium halobium, O····O, and Halobacterium salinarium, X——X, in SM (containing ortho-phosphate; 15 mg/100 ml SM).

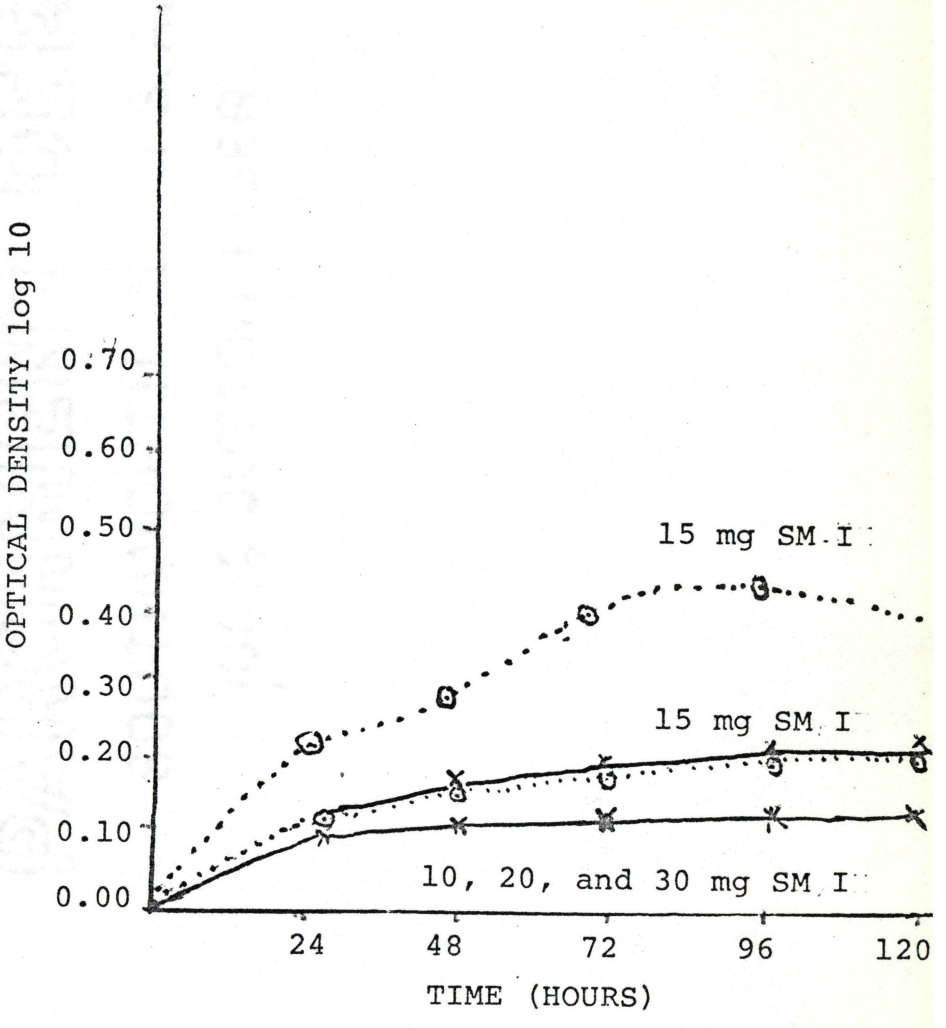


Figure 6. Growth curves of Halobacterium halobium, O····O, and Halobacterium salinarium, X——X, in SM I (containing pyrophosphate; 10, 15, 20, and 30 mg/100 ml of SM I).

(4) Effect of tripolyphosphate at various concentrations ($Na_5P_3O_{10}.10\ H_2O$: 10, 15, 20, and 30 mg/100 ml of SM II).

Tripolyphosphate caused no stimulation of growth of either species, at any concentration (Table 9 and Figure 7).

(5) Effect of DL-glycerophosphate at various concentrations (10, 15, 20 and 30 mg/100 ml of SM III).

DL-glycerophosphate at any concentration was not effective in stimulating the growth of either species (Table 10 and Figure 8).

TABLE 9: Growth of Halobacterium halobium and H. salinarium in synthetic medium containing tripolyphosphate at various concentrations of SM II.

(Mean reading of three replications)

| Concentrations tripolyphosphate mg/100 ml | Time in hours | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 24 | 48 | 72 | 96 | 120 | 24 | 48 | 72 | 96 | 120 |
| | Optical Density | | | | | | | | | |
| | H. halobium | | | | | H. salinarium | | | | |
| 10 | 0.01 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.01 | 0.012 | 0.01 | 0.01 |
| 15 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 20 | 0.01 | 0.012 | 0.011 | 0.01 | 0.01 | 0.01 | 0.011 | 0.012 | 0.013 | 0.014 |
| 30 | 0.01 | 0.01 | 0.01 | 0.012 | 0.01 | 0.01 | 0.011 | 0.012 | 0.013 | 0.014 |

TABLE 10: Growth of *Halobacterium halobium* and *H. salinarium* in synthetic medium containing DL-glycerophosphate at various concentrations of SM III.

(Mean reading of three replications)

| Concentrations glycerophosphate mg/100 ml | Time in hours | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 24 | 48 | 72 | 96 | 120 | 24 | 48 | 72 | 96 | 120 |
| | Optical Density | | | | | | | | | |
| | *H. halobium* | | | | | *H. salinarium* | | | | |
| 10 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 15 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 20 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| 30 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |

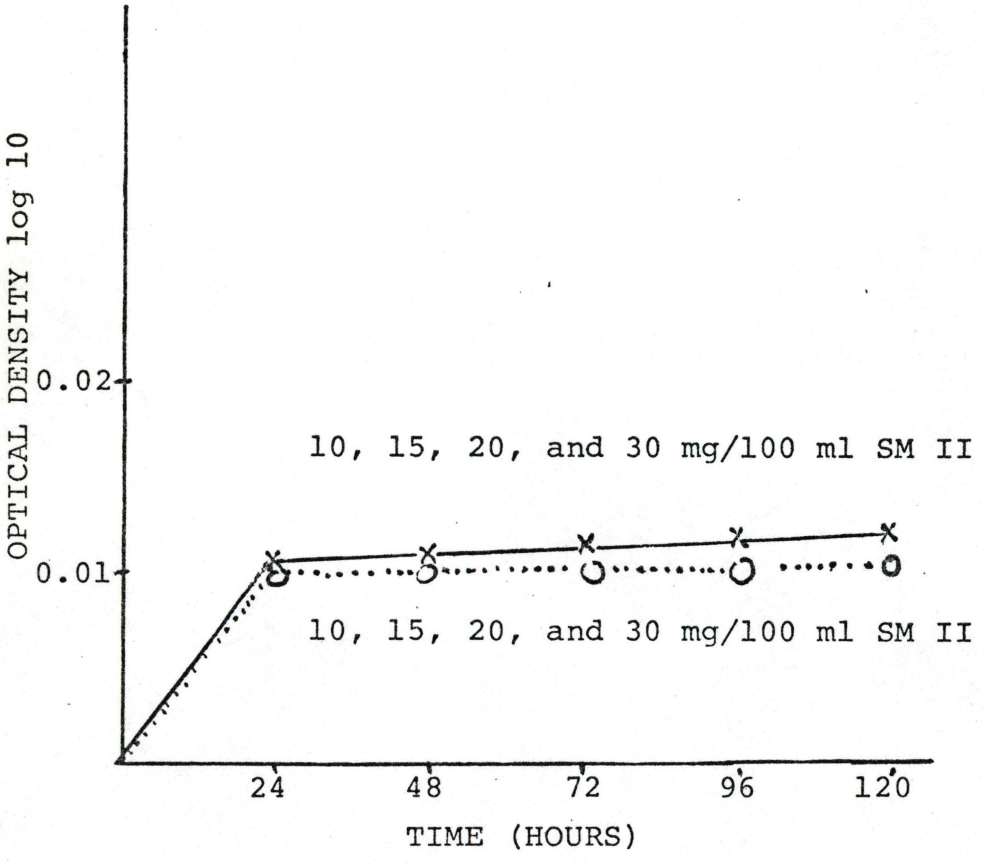


Figure 7. Growth curves of Halobacterium halobium, O····O, and Halobacterium salinarium, X——X, in SM II (containing tripolyphosphate; 10, 15, 20 and 30 mg/100 ml SM II).

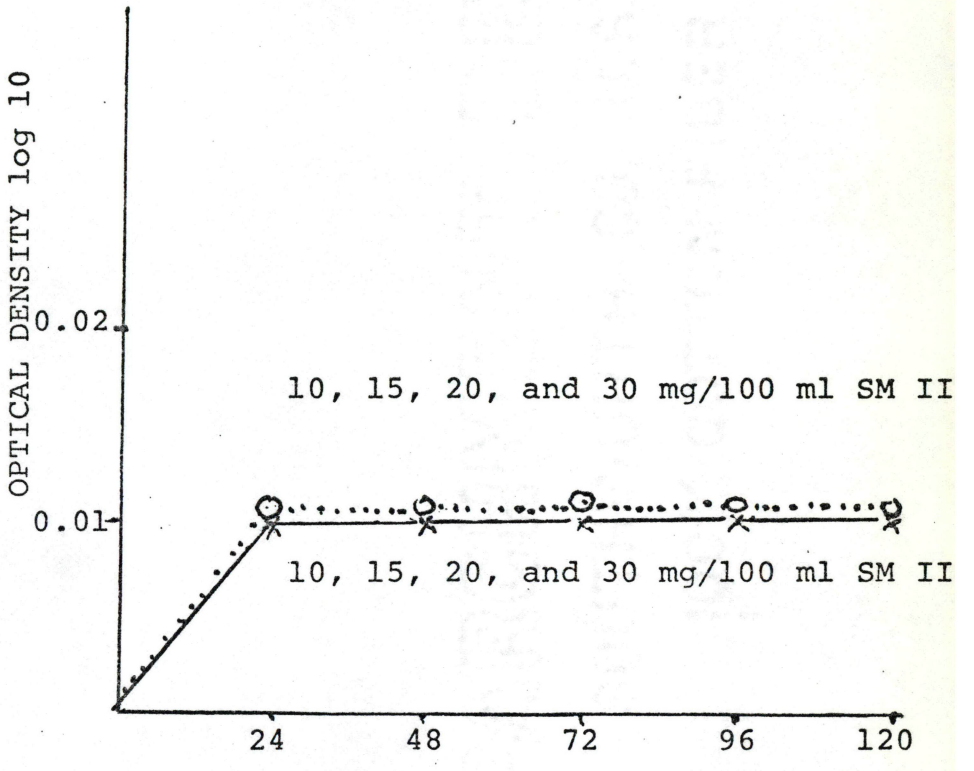


Figure 8. Growth curves of Halobacterium halobium, O····O, and Halobacterium salinarium, X——X, in SM III (containg DL-glycerophosphate; 10, 15, 20, and 30 mg/100 ml of SM III).

DISCUSSION:

Characteristics of red pigmentation, high salt requirement and cell morphology of the bacterial strains considered in this study correspond with the characteristics for halobacteria in Bergey's Manual (1974).

During the isolation procedures of the two strains, it was observed that both grew slowly on CMA and TMA surfaces and more rapidly on FPA. On the other hand, both species grew still better in CM and TM broth. The probable low oxygen solubility in the agar media (CMA, TMA FPM) with such a high salt concentration may be a reason for slow growth on those media in contrast to growth in broth media. Gouchnauer and Kushner (1969) have shown that glycerol stimulates the growth of halobacteria. Hence, the presence of glycerol in the FPA plates may have stimulated the growth of both the species. The exact nature of this effect is not known. It is suggested that the presence of glycerol provides more favorable conditions relative to maintainence of optimum surface tension of cells of both species (Gouchnauer and Kushner 1969). The stimulatory effect of glycerol is an interesting nutritional characteristic of halophilic bacteria. Dassault and Lachance (1952) noted that glycerol produced increased growth and deeper pigmentation than dextrose and other carboydrates. The cell envelope of H. cutirubrum is a largely lipoprotein and one of the main compo-

nents is a diphosphatidyl glycerol (Sehgal, et al. 1962, Kates, et al. 1963). It seems likely that glycerol is incorporated fairly readily into the cell lipids. Possibly the same is the case for H. halobium and H. salinarium. However, this hypothesis was not tested in the present study.

The temperature study on both the species indicated that the optimum temperature for growth is 37° C. This is consistent with reported optimum temperatures for other species of Halobacterium (Gouchnauer and Kushner 1969).

Growth characteristics of both species in CM, with and without calcium and in TM with and without calcium, are reported in Figures 1 to 4. It seems that H. halobium appears to grow better in all media tested (CM with and without calcium, TM with and without calcium). The mechanism for this is not known. A suggestion is that H. halobium possibly adapts easily to changes in environment. On the other hand, H. salinarium grows more rapidly in TM with calcium than in the other of the organic media. The difference between TM and CM is that TM contains tryptone (Difco) in place of casamino acids and $CaCl_2.6H_2O$. The presence of $CaCl_2$ in TM could provide stimulation of the growth of H. salinarium, but not the growth of H. halobium. It is reported that $Ca^{++}$ does not replace $Mg^{++}$ (Brown and Gibbons 1955). However, qualitative analysis of solution from which H. salinarium was isolated showed a considerable amount of $Ca^{++}$. The cultural effect of added calcium on growth of H. salinarium only in TM indicate that the calcium

and some component of the tryptone broth may act to stimulate the growth, and that the effect may be species specific.

Growth characteristics of both species in SM, SM I, SM II and SM III are reported in Figures 5 to 8. It appears that both species grow better in SM than the other modified synthetic media. The portion of this study dealing with different sources of phosphorus indicated that ortho-phosphate is the most effective source of phosphorus. Since Brown and Gibbons (1955) reported that potassium ion was essential for growth of halophiles, the presence of additional potassium ion supplied with ortho-phosphate is possibly the reason why both species grew better in SM than in the other modified synthetic media. Pyrophosphate seems to have some effect on growth of both species; specifically it has greater effect on the H. halobium than H. salinarium. It would be interesting to know why both species are affected by pyrophosphate. The effect of pyrophosphate is difficult to explain. It is possible that H. halobium adapts easily to changes in environment. Tripolyphosphate showed no effect on growth of both species.

Glycerol is reported to be stimulatory to growth of halophiles possibly as a reflection of the fact that some 73% of lipid phosphorus has been found in the di-ether analog of D-phosphatidyl glycerophosphate in of H. cutribrum (Kates, et al. 1963). The effect of DL-glycerophosphate--at various concentrations--was examined in the present study and showed no apparent stimulation of growth. It is possible that the

particles of DL-glycerophosphate do not permeate the cell membrane of either species, due to molecular size.

The primary conclusion drawn from the results gained from application of pyrophosphate, tripolyphosphate and glycerophosphate is that these forms of phosphorus have little or no nutritional value in the growth of H. halobium and H. salinarium.

## LITERATURE CITED


Baxter, R. M. and Gibbons, N. E. 1956. Effects of sodium and potassium chloride on certain enzymes of Micrococcus halodentrificans and Pseudomonas salinaria. Can. J. Microbiol. 2:599-606.

Breed, R. S., E. G. B. Murray and N. R. Smith (Eds.) 1957. Bergey's Manual of Determinative Bacteriology. 7th Ed. Williams and Wilkins Co., Baltimore.

Browne, H. J. and Gibbons, N. E. 1955. The effect of magnesium, potassium and iron on the growth and morphology of red halophilic bacteria. Can. J. Microbiol. 1:486-494.

Browne, W. W. 1947. Is salt a preservative? Biol. Rev. 9:3-6.

Buchanan, R. E. 1918. Life phases in a bacterial culture. J. Infect. Dis. 23:109-125.

Danon, A. and Stoecknius W. 1972. Photophosporylation in Halobacterium halobium. Proc. Nat. Acad. Sci. USA Vol 71, No. 4, 1235-1238.

Difco. 1953. Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical laboratory procedures. 9th Ed. Difco Laboratories, Inc., Detroit, Michigan.

Dundas, I. D., Srinivasan, V. R., and Halvorson, H. O. 1963. A chemically defined medium for Halobacterium salinarium Strain 1. Can. J. Microbiol. 9:619-624.

Dussault, H. P. 1955. An improved technique for staining red halophilic bacteria. J. Bact. 70:484-485.

Dussault, H. P. and Lachance, R. A. 1952. Improved medium for red halophilic bacteria from salted fish. J. Fisheries Research Board Can. 9:157-163.

Eimhjellen, R. 1965. Isolation of extremely halphilic bacteria. Zentralbl. Fur Bakteriol. Parasiten. Infekt. Krankheiten Ond Hyg., 1 Abt. Supp. 1:127-137.

Gibbons, N. E. 1936. Studies on salt fish. 1. Bacteria associated with the reddening of salt fish. J. Biol. Board Can. 3:70-76.

Gibbons, N. E. 1969. Isolation, growth and requirements of halophilic bacteria. In Norris, J. R. and D. W. Ribbons (Eds.). Methods in Microbiology. Acad. Press, New York 38:169-183.

Gibbons, N. E. 1974. Family Halobacteriaceae. In Buchanan, R. E. and N. E. Gibbons (Eds.) Bergey's Manual of Determinative bacteriology, 8th Ed. Williams and Wilkins Co., Baltimore.

Gibbons, N. E. and J. I. Payne. 1961. Relation of temperature and NaCl concentration to growth and morphology of some halophilic bacteria. Can. J. Microbiol. 7:483-489.

Gochnauer, M. B. and D. S. Kushner. 1969. Growth and nutrition of extremely halophilic bacteria. Can. J. Microbiol. 15:1157-1165.

Gochnauer, M. B., S. C. Kushwaha, M. Kates and D. J. Kushner. 1972. Nutritional control of pigment and isoprenoid compound formation in extremely halophilic bacteria. Arch. Microbiol. 84:339-349.

Grey, V. I. and P. S. Pitt. 1975. An improved synthetic growth medium for Halobacterium cutirubrum. Can. J. Microbiol. 22:440-442.

Harrison, F. C., and Kennedy, M. E. 1922. The red discoloration of cured cod fish. Trans. Roy Soc. Can. Sect. 5 16:101-152.

Kates, M., Sastry, P.S., and Yengoyan, L. S. 1963. Isolation and characterization of a diether analog of phosphatidyl glycerophosphate in Halobacterium cutirubrum. Biochim. Biophys. Acta. 70:705-707.

Katznelson, H. and A. G. Lochhead. 1952. Growth factor requirements of halophilic bacteria. J. Bact. 64:97-103.

Kushner, D. S. 1968. Halophilic bacteria. Adv. App. Microbiol. 10:73-07.

Lochhead, A. G. 1934. Bacteriological studies in the red discoloration of salted hides. Can J. Microbiol. 11:165-169.

Nester, E. W., Roberts, C. E., Pearsall, N. N. and McCarthy, B. J. 1978. Microbiology 2nd Ed. Holt, Rinehart and Winston, New York.

Odebela, A. 1979. Characterization and identification of an isolate of Halobacterium from Soda Springs, Mojave Desert. Unpublished thesis, California State College, San Bernardino, pp. 30.

Onishi, H., M. E. McCance and N. E. Gibbons. 1965. A synthetic medium for extremely halophilic bacteria. Can. J. Microbiol. 11:365-373.

Petter, S. 1931. Family Halobacteriaceae. In Buchanan, R. E. and N. E. Gibbons (Eds.) Bergey's Manual of Determinative bacteriology, 8th Ed. Williams and Wilkins Co., Baltimore.

Sehgal, S. N., M. Kates, and N. E. Gibbons. 1962. Lipids of Halobacterium cutirubrum. Can. J. Microbiol. 40:69-81.

Stoeckenius, W. 1964. The purple membrance of salt-loving bacteria. Sci. Ameri. 38-46.

Webber, M. M. 1949. Red halophilic micro organisms: A problem in the biochemical foundation of an ecological specialization. Biol. Rev. (City College, New York).